zone sharpening make it possible to obtain, in particular cases, much better results than when using CZE. Most promising is the combination of ITP with CZE where ITP serves as a preconcentration and pre-separation step for analysis of samples with complex matrices. Unfortunately, there is only one manual ITP–CZE system still commercially available.

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Cellulose Acetate

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The introduction of zone electrophoresis, pioneered by Konig in 1939, played a crucial role in the progress of electrokinetic separations. With this technique, molecules migrate as zones with sharp boundaries in a supporting medium immersed in a buffer solution under the application of an electric field. Zone electrophoresis was quickly found to be superior in performance to Tiselius's original technique of moving boundary electrophoresis and replaced it entirely – to be superseded in turn by displacement electrophoresis and isoelectric focusing (IEF). Interestingly, the term 'zone electrophoresis' was first suggested by Tiselius himself.

Kohn first used cellulose acetate (CA) as a supporting medium for zone electrophoresis in 1957, as a superior substitute for plain filter paper. Since then, CA has been used in many electrophoretic protocols, for both research and clinical investigations (**Table 1**). Nowadays CA electrophoresis is a widespread technique.

In this article we explain what CA is and why it is used in electrophoresis. This is followed by a brief overview of the uses of CA in various electrophoretic contexts. Finally, some recent and innovative applications of CA in electrophoretic protocols are discussed.

General Concepts

Preparation of CA

CA sheets employed in electrophoresis are made of a molecular matrix, similar in structure to a sponge but a thousand times smaller. This matrix is obtained by letting acetic anhydride react with cellulose and dissolving the product in an organic solvent, that can evaporate quickly. After letting the solvent evaporate in closely-controlled conditions of temperature and humidity, a highly permeable matrix is obtained with a uniformly distributed microporosity. The spatial volume of the pores may account for 80% of the total matrix size, ensuring ideal permeation by any

Table 1 Historical sequence of main applications of CA to electrophoretic protocols in different areas of research and clinical investigations

Year	*Application*
1957	CA is used as an electrophoretic support (Kohn)
1971	Application to conventional electrophoresis of white cell and red cell enzymes (Meera Khan)
1975	Application to isoelectric focusing of alpha-1-antitrypsin in human serum and 6–phosphogluconate dehydrogenase (Harada)
1984	Application to counterflow affinity isotachophoresis of antigens in biological fluids with low protein contents (Abelev and Karamova)
1992	Introduction of CA for protein transfer from polyacrylamide gels
1993	Introduction of protocols for reusing CA

electrolytic solution. When shaped into gel sheets CA has better resistance to the dehydration involved in the dissipation of heat and is more easily handled. Thus, pre-gelled CA membranes (also referred to as Cellogel™) are the first choice of support for many electrophoretic applications. For better handling, some commercial versions of Cellogel™ come welded to an inert support of polyester plastic (Mylar™). These commercial forms of CA pass practically unchanged through the entire separation–staining–destaining cycle of a classical electrophoretic experiment.

There are several major factors accounting for the versatile electrophoretic properties of CA: (1) the cellulose chain length, which ranges from a few to millions of individual molecules; (2) the degree of acetylation (from 0.1% to 44%); (3) the pore size (between 50 Å and 10 μm), the random pore distribution and the volume of the pores compared with the solid matrix (20% to 80%). The spatial coiling of cellulose molecules, the type and concentration of wetting agents and the presence of residual contaminants may also be important factors.

CA as an Electrophoretic Medium

Migration of molecules through the CA matrix depends mainly on the nett charge on the molecule, the buffer pH and ionic strength and the intensity of the electric field. The difference in surface nett charge between the molecular species in a sample to be separated is perhaps the most important point to consider. Proteins are amphoteric, like their constituent amino acids, and they may be charged positively or negatively depending on the pH of the solvent medium (the buffer solution, in an electrophoretic experiment).

In gel electrophoresis a sieving effect may affect the separation, depending on the critical relationship between the spatial shape of a protein species and the pore size of the matrix medium. Because of the extremely large cellulose matrix pores, the mobility of proteins in CA electrophoresis is a direct function of their surface net charge, whereas molecular weight and shape are less important. For example, the human heavy α-2 macroglobulin (M_r: 1 000 000; pI 5.9) moves faster than the much lighter haptoglobin (M_r 100,000; pI 6.1) in alkaline buffer solutions.

As in most electrophoretic protocols, to improve a CA separation the ideal buffer pH and ionic strength, strip temperature, voltage, current, electroosmosis and time of separation should be selected. The optimal ionic strength is between 0.01 and 0.1 (mequiv L^{-1}). Although mobility is theoretically enhanced at high temperature, proteins are easily heat denatured so the separation temperatures should be kept below 50°C. Moreover, since CA electrophoresis is traditionally carried out with no cooling, separation voltages should not exceed 500 V (60 V per linear centimetre in gel strips), and the current should be adjusted to less than 2.5 mA cm^{-2}. CA contains polar groups – hydroxy (OH^-) and acetyl (CH_3COO^-) radicals – that become charged at the pH system and move towards the anode through the cellulose matrix. This produces a counter-reaction, displacing buffer toward the cathode and interfering with the separation of the molecules of interest (endoosmosis). Prolonged separation times may thus lead to the creation of artefacts due to the combined effects of heat, buffer turbulence and the counter-diffusion of molecules. Running times should be altered accordingly.

A few fundamental properties make CA electrophoresis notably superior to electrophoresis using filter paper: (1) the CA matrix is homogeneous, microporous and chemically pure, reducing molecular adsorption to a minimum; (2) instant heat dissipation occurs in the matrix, which does not need to be cooled; (3) the amount of protein needed is very small (1 mg or less); (4) the inherent buffering–staining–destaining system is very simple; (5) stained CA strips have no background; (6) the standard electrophoretic apparatus required is simple and inexpensive (**Figure 1**).

For most purposes – especially for routine clinical investigations – small-scale CA electrophoresis (with membranes < 10 cm long) is widely used (**Figures 2** and **3**). Larger scale membranes (usually 20 cm long) suit a variety of research analytical purposes and micropreparative applications.

CA in Electrophoretic Protocols

Conventional Electrophoresis

CA was originally introduced as a classical support for analytical zone electrophoresis but found a much broader range of applications. Essentially, it can now be used for both analytical and preparative purposes. Preparative applications exploit the speed of CA separations, the absence of molecular interaction, and the easy recovery of biologically active substances from the matrix.

CA is popular in clinical laboratories in which some well-established routine analyses are performed, e.g. for haemoglobin, serum proteins, lipoproteins and lactate dehydrogenase. Isoforms of many enzymes and proteins from different tissues come out very clear-cut on CA – a fact that is (or has been) of particular interest for anthropogenetic and

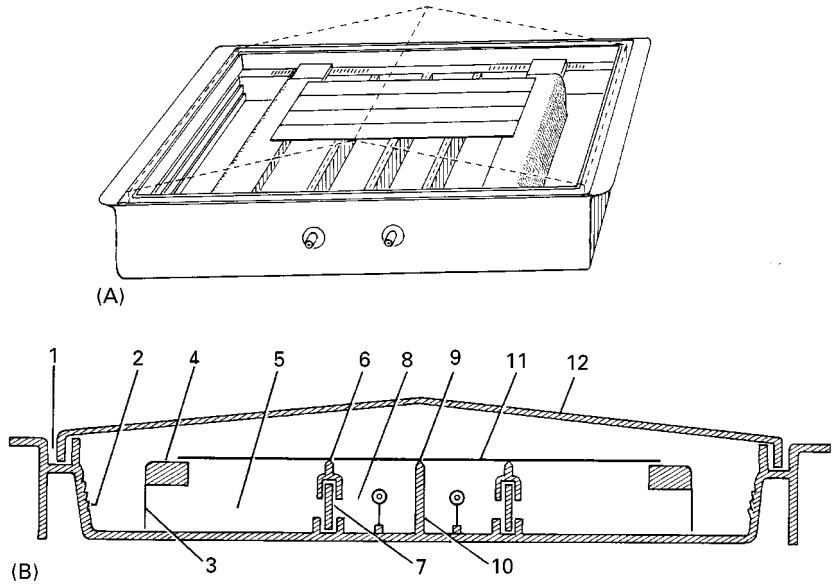

Figure 1 Description of a universal electrophoretic apparatus for CA electrophoresis (redrawn and modified from Kohn, 1957). The CA strips (11) are supported at each end by the shoulder pieces (4) and when taut are just clear of the top edge of the centre partition (10). The top edge of this centre partition is formed as a row of pyramids (9) which support the strip should it tend to sag. When using long strips, strip supports (6) may be fitted to the labyrinth partitions (7) that form the connections between the buffer compartments (5) and electrode compartments (8). Filter paper wicks (3) connect the CA strips to the buffer compartments. The internal sides of the tank are stepped all round (2) as an aid to buffer level checking. The lid (12) fits in a recess (1) moulded all round the tank.

forensic purposes and for the biochemical characterization and classification of various pathogenic microorganisms such as *Leishmania* and *Trypanosoma* species.

In addition to one-dimensional electrophoretic methods, two-dimensional CA electrophoretic protocols are also available. A summary of important applications is given in **Table 2**.

Detection and Quantitation

Any protein stain can be used with CA, provided that the solution does not contain a cellulose solvent. Aqueous staining solutions are preferred to alcoholic ones, since with the latter strips tend to shrink and curl unless they are passed through an aqueous bath. Staining solutions for CA are less concentrated than those used in filter paper electrophoresis, and they can be repeatedly used with no appreciable loss of sensitivity.

A wide range of analytical applications can be listed with an impressive variety of fully compatible staining methods, including Coomassie blue brilliant, Ponceau red, Nigrosin, Schiff, gold and silver stain, different types of immuno-staining, and many different types of enzyme specific staining. A 5% (w/w) aqueous solution of acetic acid is a universal washing solution for reducing the background.

The simplest way of evaluating the results is by visual inspection of stained strips, which should be carried out against a strong light source to improve the assessment of the separation pattern.

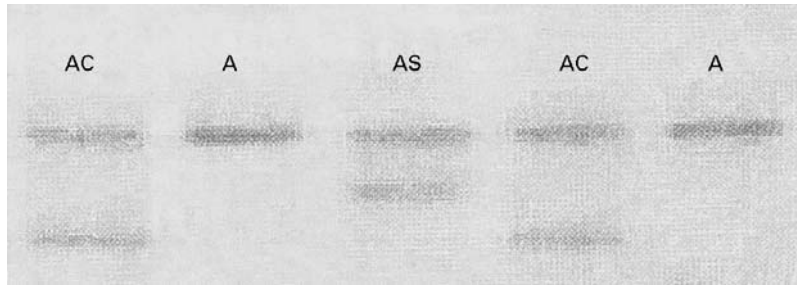

Figure 2 Electrophoretic separation of human haemoglobin variants A, C and S. Ponceau red staining was used to visualize hemoglobin bands, and the anode was on top.

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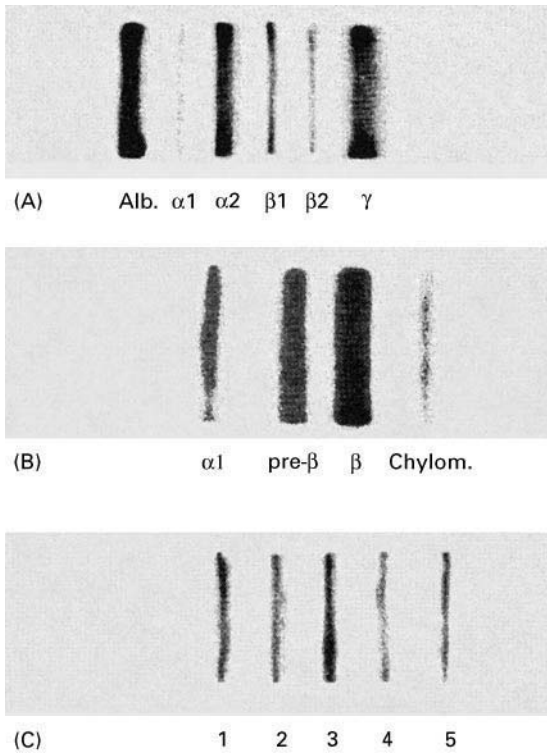

Figure 3 Routine clinical electrophoretic separations on CA: (A) serum proteins; (B) lipoproteins; (C) Lactate dehydrogenase isoenzymes. Samples were obtained from healthy patients.

Quantitative determinations can be carried out by elution or by scanning of the stained strips. Once stained, protein bands can be easily eluted from the membrane by an appropriate buffer system (a classical system is Tris (2-amino-2-hydroxymethyl-propane-1,3–diol) or Barbitone elution buffer over Ponceau red stained bands). Alternatively, a solvent (e.g. chloroform–ethanol 9:1 v/v) can be used to dissolve the membrane and recover the protein of interest. To enhance the recovery efficiency, gelled CA blocks (about 0.5 cm thick, instead of much thinner 0.5 mm supports) can be used.

Table 2 Some recent applications of CA electrophoresis

Year	*Application*
1994	Introduction of thermocooling apparatus for CA IEF
	Sequential electrophoresis, with detection of 21 different alleles in ESD-2 locus in *Drosophila buzzatii*
1995	Improved separation of apolipoproteins by use of surfactant Tween 20
1996	Rapid screening of biochemical loci of rat
	Highly sensitive detection of urinary proteins using colloidal silver staining
1997	Detection of superoxide dismutase isozymes to distinguish between *tsetse* blood meals of human and non-human origin
	CA electrophoresis used as the method of choice for alpha-thalassaemia screening
	IEF on CA applied to the analysis of microheterogeneity of immunoglobulins and serum protein fraction

Scanning is preferred to elution for routine clinical applications. To reduce background and increase sensitivity, CA strips should be cleared prior to scanning. As with filter paper it is important to use oil with the same refractive index as the support. CA strips cleared with oil may be returned to their original dry state by using a solvent such as ether. By contrast, swelling agents such as acetic acid and dioxan used in conjunction with heat treatment, permanently clear CA.

Isoelectric Focusing

CA has ideal features to suit IEF separations. CA is virtually a non-sieving matrix enabling a quasi-free fractionation of macromolecules according to their respective isoelectric points (p*I*, the pH at which there occurs an equal number of negative and positive surface charges). CA is easily soaked with very small amounts of carrier ampholyte species, allowing them to be eluted in due course with no damage to stained–destained proteins; this in turn allows densitometry measurements and storage.

Unfortunately, the combined effect of CA electroosmotic flow and the low ionic strength of commercial ampholines can seriously impair the resulting separation of proteins at their isoelectric points. To overcome these drawbacks, CA has been variously treated with surface active agents or with methylating agents. Such treatments can partly – if not wholly – reduce the osmotic flow. Also, a high concentration of carrier ampholytes should be used to cover broad pH ranges (8% v/v instead of the customary 2% v/v) and electrolyte additives at low concentration (such as 0.2 M lysine and 0.2 M acetic acid) should help stabilize narrow pH intervals. Untreated CA strips give better results when 5% β-mercaptoethanol and 5 M urea are used as stabilizing agents.

Alternative strategies to circumvent electroosmosis, which differ in effectiveness, involve shortening the inter-electrode distance or using 'chemical spacers' to flatten the pH gradients at the appropriate segment of separation. These devices may help to create high field strengths with low voltages. Recently, thermoelectric cooling has been used to stabilize CA IEF gradients.

Counterflow Affinity Isotachophoresis

Isotachophoresis or 'displacement' electrophoresis permits simultaneous concentration and effective separation of surface-charged substances, including biological macromolecules. With this analytical method,

proteins are stacked as closely spaced, narrow bands between the 'leading' and the 'trailing' ions. Isotachophoresis on CA gels takes advantage of the absence of sieve effect in this matrix to study sets of interacting biological macromolecules, such as antigen/antibody and glycoprotein/lectin systems. However, electroosmosis once again interferes with this application. Abelev and Karamova were able to overcome this drawback by demonstrating that the cathodic counterflow, combined with the constant flow of liquid through the membrane, stabilizes separations. The counterflow may be also used as a 'conveyer belt' to move immunoreagents through antigens or antibodies immobilized onto the membrane. Abelev and Karamova used a discontinuous buffer system, in which the two buffers have the same cation and differ in the anion species (chloride as the leading ion and β-alanine as the trailing ion). Under these conditions, macromolecules are separated between the two anions.

Abelev and Karamova's method was originally developed to analyse proteins in highly dilute biological fluids such as urine, tears, and cerebrospinal and amniotic fluids, and it turned out to also be useful for detecting low levels of urinary monoclonal immunoglobulin light chains (Bence Jones protein) and alpha-fetoprotein in various pathological conditions.

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CA as a Reusable Electrophoretic Support

CA separations are faster than those on other supports, usually with no resolution loss. However, CA sheets cost considerably more than starch, agar, agarose or polyacrylamide gel sheets.

Recently, a wash method has been described that makes it possible to recycle CA strips. The procedure has been shown to work even after using the strips for analysis of a variety of erythrocyte isoenzymes, which notoriously expose the support matrix not only to the strain of the electric field but also to many somewhat elaborate biochemical colorimetric treatment steps. Surprisingly, none of these stages seem to irreversibly affect the mechanical and physicochemical properties of the CA. In fact, after a variety of enzyme activity tests (adenosine deaminase, adenylate kinase, carbonic anhydrase, erythrocyte acid phosphatase, esterase D, glutathione peroxidase, glyoxalase 1, phosphoglucomutase and 6-phosphogluconate dehydrogenase) Cellogel™ returns to its original features if soaked/washed in water and methanol for a short time. In the course of double blind trials, no difference in band sharpness and resolution was noticed between new and used Cellogel™ strips. The procedure can be repeated two or three times if care is taken to avoid warping strips with absolute methanol soaking or rough handling.

Blotting Proteins from Polyacrylamide Gels to CA Sheets

Different electrophoretic species run in the same gel for the same time with the same electric field settings. The end of a given experiment is currently set depending on the specific requirements of the molecules to be separated, in zone electrophoresis as well as in IEF.

To achieve optimal resolution of different protein constituents of the same sample, various experiments are often carried out, only differing in voltage and duration. To save time, a simple method involves repeatedly blotting a polyacrylamide gel with CA sheets at various stages of separation. The blots obtained in this way can be stained and the protein species made to show the optimal resolution.

The advantages that can be obtained from CA blots of the same acrylamide gel are great, the most outstanding being:

1. various stages of a single protein separation can be tested in one experiment, to improve the protocol;
2. common and rare variants of a single electrophoretic pattern can be detected, each under optimal separation;
3. several proteins can be analysed at optimal conditions in the same experiment;
4. all the allele products may be discriminated by isotacophoretic mechanisms (in non-equilibrium IEF) and isoelectric point (in true equilibrium IEF) within the same run.

Conclusion

Almost uniquely among the various supports for electrokinetic separations, CA electrophoresis is still intensively used for both research and routine applications. The reasons for this long-lasting success are clear: simplicity of use, low cost, versatility and cost effectiveness. These same factors are likely to provide the general basis for the continuing use of CA in the future.

Acknowledgement

The drawing of Figure 1 was provided by Niccolò Falchi of the Department of Animal and Human Biology, University of Rome 'La Sapienza'.

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Deoxyribonucleic Acid, Theory of Techniques for Separation

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Introduction

Separation of biochemical molecules can be carried out in gels or polymer solutions and, in specific cases, in free solution, using constant or variable electric fields. Gels are used primarily in deep-dish containers, submerged in buffer, and polymer solutions are used in glass capillaries, with inner diameters less than 100 μm. Thin gels between two glass plates have been used for separating and sequencing single-stranded DNA molecules. We begin the theoretical discussion by considering the separation of double-stranded DNA molecules (dsDNA) in submarine gels under constant electric field conditions.

Geometrical Sieving Model for Small DNA Molecules in a Constant Electric Field

Ogston was the first to calculate the fractional volume available to a sphere of radius R in a gel of a given concentration. The gel itself was modelled as a random array made up of fibres of radius r. Within this description, a sphere with a radius $R \gg r$ cannot pass through the network if the sphere is not allowed to deform. This geometrical model predicts that the electrophoretic mobility of DNA molecules, as a result of molecular 'sieving', varies as:

$$\frac{\mu}{\mu_0} \propto \exp\left[-\left(\frac{R_g}{a}\right)^2\right] \qquad [1]$$

where R_g is the radius of gyration of the DNA molecule, μ_0 is the free solution mobility, a is the average pore size of the gel, and the exponential dependence of the mobility arises from the assumption of Poisson statistics for the distribution of spaces in a random network of straight fibres. This model describes the mobility of small molecules when they first encounter the gel fibres as obstacles to molecular motion. The analysis of experimental data using eqn [1] is highly model dependent, but can provide some guidance for the development of new gel structures for more efficient electrophoretic separations of small molecules.

Entropic Trapping of Small DNA Molecules

For DNA in the entropic size regime, the deformable molecules select the larger pores in order to maximize locally their conformational entropy. However, in order to accomplish this, they must squeeze through the narrow channels connecting the larger pores. The corresponding polyelectrolyte dynamics is dominated by an activation process in this regime, where the electrophoretic mobility is given by an inverse power law (> 1) over a size range that is larger than for the Ogston regime, but smaller than for the beginning of reptation, which is discussed in the next section.

Gel Electrophoresis of Large DNA Molecules in a Constant Electric Field

Figure 1 shows a schematic picture of a gel matrix, in which a DNA molecule is embedded. For a molecule that is much longer than the average spacing between the chemical cross-links of the gel fibres, the molecule cannot move through the gel as a random coil, rather